STAPHYLOCOCCAL HYALURONIDASE

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Thesis

Staphylococcal Hyaluronidase

by

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I. INTRODUCTION

The purpose of the present investigation is to study the production of the enzyme hyaluronidase by the staphylococcus, and to demonstrate a method whereby a partial purification of this enzyme may be effected. The activity of the enzyme is measured by a turbidimetric method.

II. BODY OF THE THESIS

1. History and Current Investigation of Hyaluronidase

The enzyme now known as hyaluronidase was first described by Duran-Reynals in 1928. (15) (75). He found that vaccinial infection in shaved rabbit skin is extraordinarily enhanced by the simultaneous injection of testicular extract. Duran-Reynals later found what we now term spreading factors in invasive bacteria, (17) in poisonous insects and in snake venoms. (18). Work of a similar nature was done by McClean. (46) (47).

In 1934 the work of Carl Meyer and his associates revealed the true nature of material previously classified as "mucoprotein" or "glycoprotein." These workers discovered in the vitreous humor a nitrogen-containing polysaccharide which they termed Hyaluronic acid - hyaloid (vitreous) + uronic acid. (55). Later similar polysaccharides were found in umbilical cord, (56) in synovial fluid (59) and in many other parts of the animal body. Hyaluronic acid itself is considered as consisting of entangled chains, each of which consists of

disaccharide units, many times repeated, the disaccharide being formed of 2-acetylamino-glucose and glucuronic acid. The characterizing property of the product is its viscosity, although this property varies with the source and preparation. Meyer and his group obtained from pneumococci an enzyme which was capable of hydrolyzing three polysaccharide acids of apparently identical structure, obtained from vitreous humor, umbilical cord and Group A streptococci. (58)

In 1939, Chain and Duthie suggested that "spreading factors" were hyaluronidases. (6). They termed the enzyme "mucinases" and showed that their preparations would, successively, abolish the power of hyaluronic acid to coagulate with protein in acid solution, reduce the viscosity of hyaluronic gels and solutions, and eventually set free N-acetyl glucosamine indicating complete hydrolysis.

Hyaluronidase inhibition has been investigated. Duran-Reynals showed that the spreading factor disappeared from the blood after intravenous injection. (17). Hobby and co-workers found that normal human and rabbit sera inhibited the action of hyaluronidase prepared from cl. perfringens and certain streptococcal strains. (33). McClean found that hyaluronidases prepared from bull, rabbit and mouse testes were inhibited by guinea pig, rabbit, sheep, horse, mouse and human serum; also that heparin, chondroitin sulphate and gastric mucin had an inhibitory action. He did not consider the inhibitor in blood indentical with any one of these substances,

since its chemical behavior indicated that it was a pseudo-globulin. (50). The defense mechanism of the body against the action of hyaluronidase was first described in a series of papers by Haas. (26) (27) (28). From his data on reaction rates and effects of temperature changes he concluded that hyaluronidase is inhibited *in vivo* by an antienzyme which he termed antinvasin I. He described a complex system consisting of at least two different antinvasins and proinvasin. Recently it has been suggested (10) that this work was done with crude hyaluronidases and no account was taken of the possible effects of contaminating enzymes. Hadidian (30) believes that Haas' concept of hyaluronidase inhibition by an enzyme is untenable since the hyaluronidase acitivity which is initially lost on incubation with serum is recovered by prolonging the incubation without altering the experimental conditions. He also found that the inhibitor was a protein and suggests that it may be conjugated with an oligosaccharide. Dorfman *et al* (10) have devised a method for the estimation of the inhibitor in human blood. He (11) points out that since hyaluronidase inhibition can be shown in a variety of species irrespective of previous exposure to hyaluronidase the inhibitor is not an antibody in the usual sense.

The role of hyaluronidase in effecting fertilization was first shown by McClean and Rowlands, (51) who discovered that hyaluronidase present in semen dissolves the cementing material which connects the cumulus cells surrounding the tubal

ova of the rat. This allows the spermatazoa to effect fertilization. A similar observation was published almost simultaneously by Fekete and Duran-Reynals. (20). These workers found that crude or highly purified preparations known to be very rich in hyaluronidase, such as extracts from rattlesnake venom, leech tissues and testicle have a very pronounced effect in dispersing the follicular cells surrounding the ova of mice. Later work by Rowlands (70) showed relationship between intromission of such a large number of sperm and the establishment of the requisite concentration of the enzyme in effecting fertilization. He found that in rabbits 1×10^6 or more sperm are required for maximum fertilization; only a small number of eggs are fertilized when the inseminate contains 2×10^5 sperm; 1×10^5 sperm are probably incapable of causing fertilization. The addition of a sperm-free hyaluronidase filtrate to a semen sample containing too few sperm to effect fertilization resulted in 4 out of 5 successful fertilizations. Werthessen et al (78) and Bergenstal and Scott (3) have shown that human sperm counts are roughly proportional to the seminal hyaluronidase levels. Clinical application of these observations has recently been shown by Kurzrok (40) who studied a series of 315 cases treated for human infertility. Alternate patients were treated by instillation of the enzyme into the cervical canal on the calculated date of ovulation, irrespective of the hyaluronidase content of the mate's semen. Results are as follows:

Total cases - 315

Total Conceptions - 59 (18.8%)

A. Treated with hyaluronidase - 158 cases

Conceptions - 42 (26%)

B. No hyaluronidase applied - 157 cases

Conceptions - 17 (11%)

Kurzrok contends that in the treatment of human infertility by any method or combination of methods, to obtain as high as 15% of conceptions is considered good. Chambers and Zweifach (7) have found that an extract of sea urchin sperm caused great swelling and softening of the jelly coat of sea urchin eggs. The extract did not effect the intercellular cement which holds together the blastomeres of the developing egg, thus presumably precluding the possibility of producing a monster. The specificity of the hyaluronidase is indicated by the fact that an extract from bull sperm had no effect on the jelly coat of the sea urchin egg.

Various investigators have been interested in the hyaluronic acid - hyaluronidase system in bacteria and its relation to invasion and virulence. Kendall and co-workers (38) demonstrated hyaluronic acid in the culture media of three types of Group A hemolytic streptococci in the mucoid phase and Seastone (73) isolated hyaluronic acid from Group C hemolytic streptococci in the mucoid phase. McClean (54) demonstrated that capsules and hyaluronidase cannot co-exist in the same Group A or C strain of streptococci. He also showed that 94%

of strains from moderate or severe streptococcal infections in man have been found to produce the mucoid polysaccharide in greater or less amounts. In a group of streptococci from normal throats only about 8% produced hyaluronic acid, all of the producers falling into Lancefield's Group A. Recent work on this subject has been done by Sallman and Birkeland. (71). These investigators found that hyaluronidase-producing strains of Group A streptococcus were more virulent for the chick embryo than were non producing strains. They studied the utilization of hyaluronic acid and its hydrolytic products in aerobic respiration by three strains of Group A hemolytic streptococci:

Griffith type 2 Griffith type 9	Producers of hyaluronidase only when hyaluronic acid added to media; low virulence for chick embryo.
Griffith type 10	High hyaluronidase producer; highly virulent for chick embryo.

All three strains could use hyaluronic acid for respiration, types 2 and 9 producing a 3 to 4-fold increase in oxygen uptake in its presence, as compared with a 12-fold increase for type 10. Type 10 was used to prove their hypothesis that the products of hydrolysis rather the substrate were being utilized in respiration. Their results showed that glucuronic acid stimulated oxygen uptake only slightly while N-acetyl glucosamine gave a maximum 18-fold increase; glucosamine itself was subsequently found to stimulate oxygen uptake even more than the N-acetyl derivative. On the basis of this work

Sallman and Birkeland suggest the following explanation of the manner in which hyaluronidase fuctions in streptococcal virulence:

> "Production of hyaluronidase by virulent strains makes available to them for energy pruposes some of the large amount of hyaluronic acid normally present in the host. In addition since glucosamine is readily utilized by a large number of organisms, this function of hyaluronidase may well explain the numerous observations that other disease-producing agents are frequently found in association with hemolytic streptococci."

Frious (21) has shown that pooled human gamma globulin contains an inhibitor exhibiting a specific effect against Group A streptococcal hyaluronidase; also that in the majority of 50 cases of scarlet fever, the titre of this inhibitor increased. The hyaluronic acid-hyaluronidase system in clostridia has been studied by McClean (48). This author found that hyaluronidase is produced by organisms of the gas gangrene group and that the inclusion of potassium hyaluronate in the culture medium of cl. perfringens resulted in an increased production of the enzyme by the organism. (49). From this fact he postulated that the presence of hyaluronic acid in vivo increased enzyme production, setting up a vicious circle which promotes rapid extension of the infection. Duran-Reynals (17) correlated invasiveness of strains of staphylococci and streptococci with the yield of diffusing factor.

Guerra, (24) (25) basing his observations on the work of Meyer and Palmer on hyaluronic acid, (57) first pointed out a possible relationship of hyaluronidase to rheumatic fever. He

observed that this is a disease of the mesenchyme, characterized by its invasiveness, and that the principal regions effected, such as articulations and synovial fluid, are largely composed of hyaluronic acid. He found that in people who had acute rheumatic fever or who gave histories of an attack, intracutaneous injections of hyaluronidase with Evans blue gave large and abnormal diffusion of the dye and enormous local areas of edema. Salicylates in these cases inhibited the enzyme and reduced its specific effect in connective tissues. He also found that sodium salicylate caused a reduction in the skin diffusion effect of hyaluronidase in rabbits, and concluded that the anti-rheumatic action of salicylates could be explained by their enzyme-inhibiting action. Subsequent work by Swyer (76) has shown that *in vitro* sodium salicylate and acetyl salicylate effect the viscosity-reducing action of hyaluronidase only in relatively enormous concentrations. This author suggests that the hyaluronidase preparation used by Guerra might have been contaminated with histamine or a histamine-like substance and that the inhibitory effect demonstrated may have been due to the anti-histamine property of these drugs. This view is in accord with the work of Hechter (32) who showed that extent of spreading in the skin by hyaluronidase is greater when capillary permeability is not increased. Dorfman *et al* (9) have found that sodium salicylate inhibits the specific effect of hyaluronidase *in vivo*. Hyaluronidase derived from *cl. perfringens* and from bull testis is also inhibited by

sodium salicylate *in vitro*, but the concentration required for *in vitro* inhibition is considerably larger than that obtained *in vivo*. In this connection it is interesting to note that it has been shown by various workers (1) (34) (2) (4) that sodium salicylate at high concentrations causes a reversible denaturation of certain biologically active proteins. Experiments by Pike (64), also by Lowenthall and Gagnon (42), using relatively low concentrations of sodium salicylate failed to show any inhibitory effect *in vitro*. The latter workers found gentisic acid (2, 5 dihydroxybenzoic acid) also inactive, but showed that its quinone, carboxy-p-benzoquinone, was inhibitory at a relatively low concentration. Ragan and Meyer (65) report that gentisic acid has been found to possess antirheumatic properties in human beings, whereas Dorfman (12) claims that pure gentisic acid shows no inhibition *in vitro*. Guerra has continued to investigate the inhibition of the spreading effect in the skin (22) (23) and apparently maintains his original conclusions. From the foregoing statements it is evident that the present concept of the role of hyaluronidase in rheumatic diseases and its relationship to salicylates and their possible metabolites is somewhat confused.

Duran-Reynals and Stewart (16) first investigated tumor tissue as a possible source of hyaluronidase. They found that aqueous or Ringer's solution extracts of 28 human epithelial tumors enhanced vaccine virus infections in 11 cases, inhibited it in 10 and had no effect in 7. Their extracts of

13 human sarcomata showed no enhancement of spreading and in 10 of the cases, definite inhibition. A small series of similar extracts from transplantable animal epitheliomata and sarcomata gave comparable results. Boyland and McClean (5) investigated the spreading factor content of a large number of animal tumors ranging from benign tumors to invasive neoplasms. They concluded that the spreading factor content paralleled the malignancy of these tumors. Coman, McCutcheom and Zeidman (8) used a highly malignant mouse sarcoma and the potentially malignant Shope rabbit papilloma to show whether hyaluronidase could enhance invasiveness. They found that it did not promote local invasiveness or metastasis. In contrast, Simpson and Gopal-ayengar (74) reported that the local injection of testicular hyaluronidase caused a notable increase in the growth and invasion of a transplantable mouse squamous cell carcinoma.

Recent clincial applications of hyaluronidase are varied. It has been used in nerve blocks, to enhance the spread of procaine. (39). Narins et al (63) have studied the effect of hyaluronidase on urinary calculi. Five of 8 urinary calculi immersed for one hour in hyaluronidase solutions showed considerable fragmentation; none of their saline controls did. Meyer (61) reports on an investigator who found that the injection of approximately 100 turbidimetric reducing units of purified testicular hyaluronidase into a rabbit's eye appeared to liquefy the vitreous humor, and speculates that the cause of

simple glaucoma may well be explained by inhibition of hyaluronidase in the eye.

2. Quantitative Assay of Hyaluronidase

Hyaluronidase may be assayed by various methods:

(1) Measurement of the "spreading effect" in the skin of animals, using as an indicator a substance such as India ink. (43) (35).

(2) Mucin Clot Prevention Method. (66) (52).

This method is based on the observation that native hyaluronic acid and acidified protein form a typical fibrous "mucin" clot. After incubation with hyaluronidase the quantity of the clot is reduced and the character of the precipitate changes, depending on the amount of enzyme used. The error of this test has been estimated as ± 25 per cent. (53).

(3) Groups A and C streptococcus Decapsulation Method (50)

Here the substrate is the capsule of the organism and the amount of enzyme necessary to effect decapsulation is calculated. The objection to this method is that these organisms sometimes lose their capsules under various conditions, without the addition of enzyme. (61).

(4) Viscosity Reducing Method. (44)

By this method the time required for the hyaluronic acid to reach half viscosity is found to be inversely proportional to the concentration of hyaluronidase present. This method is accurate and has been used widely. It is, however, tedious and time-consuming and requires large amounts

of hyaluronic acid. One unit is defined as the amount of enzyme required to reach half viscosity in 30 minutes.

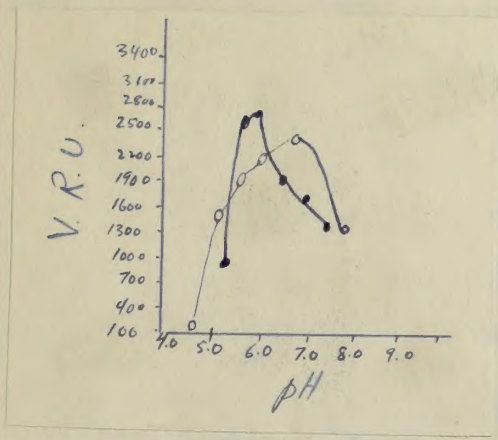
(5) Measurement of increase in reducing sugar, or by the increase in liberated N-acetyl glucosamine.

This requires that pure hyaluronate of known hexosamine and uronic acid content be used. In using this method Hahn (31) found that highly purified hyaluronidase does not bring about the complete hydrolysis of hyaluronic acid. He believes that contaminating enzymes in the crude preparation bring about the release of glucuronic acid and N-acetyl glucosamine. Humphrey (36) confirmed the discrepancy between reducing and N-acetyl glucosamine values on hydrolysis of hyaluronate, using an excess of each of 4 different enzyme preparations. The work of Rogers (69) has clarified this matter. This worker used a staphylococcal and streptococcal strain to compare the influence of pH on:

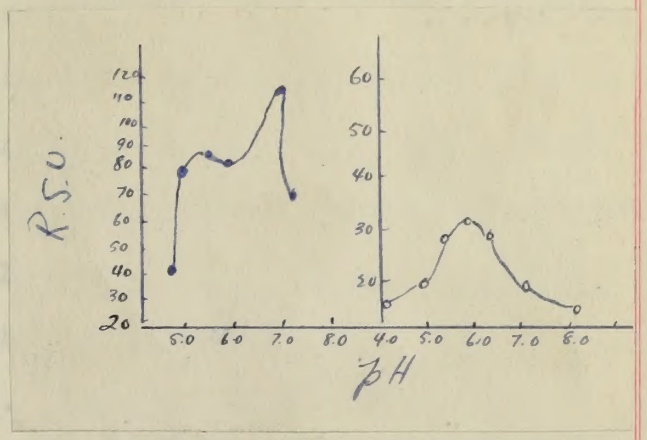
(a) The viscosity-reducing activity.

(b) The amount of reducing sugar liberated.

The results of this work are shown in the graphs on Page 13.



Influence of pH on Viscosity-Reducing Activity of Hyaluronidase from Strains of Strep. and Staph.



STREP.
STAPH.

Influence of pH upon Amount of Reducing Sugar Liberated from Potassium Hyaluronate by Hyaluronidase upon Strains of Strep. and Staph.

Rogers states that the double optimum obtained with the streptococcal enzyme suggests that more than one enzyme is involved in the liberation of reducing sugar. It may be significant that one of these optima agrees with the optimum at pH 5.7 obtained by viscosity reduction. The difference between the optima for the staphylococcal preparation is a whole pH unit, suggesting the existence of more than one staphylococcal hyaluronidase.

(6) Turbidity Reducing Method

Seastone (73) showed that the addition of hyaluronic acid to acidified protein at controlled pH and ionic strength will result in a turbidity which is proportional to the amount of hyaluronic acid present. Kass and Seastone (37) later showed that after incubation with

hyalurondiase, under specified conditions, no turbidity develops on addition of acidified serum to the hyaluronic acid. This is the principle upon which the quantitative turbidimetric method is based. Meyers (62) points out that this method is apparently based on the hydrolysis of the N-acetyl-glucosaminidic linkage.

Various modifications of this method have been used by other workers, (41)(61)(13)(77). In the present study an unpublished turbidimetric method made available by the Schering Corporation was used.

The turbidimetric method may be applied to the estimation of hyaluronidase in 2 ways:

(1) By determination of the rate at which hyaluronic acid is hydrolyzed. This method was used by Dorfman (14) to study the kinetics of the reaction. By taking into account changing substrate concentrations he concludes that this is a first order reaction under the conditions studied.

(2) By determination of the amount of hyaluronic acid which remains after some specified time.

The Schering Method falls into Group (2).

A paper by Dorfman and Ott (13) emphasizes that in the turbidity method certain factors effect hyaluronidase activity:

(1) pH - activity drops more rapidly on the alkaline side of the maximum than on the acid side.

(2) ionic strength - increasing ionic strength causes a decrease in the activity of the enzyme.

McClean and Hale (49) as well as Hadidian and Pirie (29)

believe that the chloride ion is an important activating agent. These two groups of workers used the viscosity reducing test. Hadidian and Pirie studied the effects of several salts on the rate of viscosity reduction, keeping pH as constant as was possible with low salt concentration. They concluded 3 groups are distinguishable:

(a) $Cacl_2$ and $Mgcl_2$ - effective in very low concentrations.

(b) Nacl, Kcl and NH_4cl - just as effective but in higher concentrations.

(c) Na_2SO_4 and Na_2HPO_4 - relatively ineffective.

Sodium acetate is intermediate between chloride and phosphate. These workers obtained the optimal concentration for each ion at a given pH. Dorfman and Ott (13) point out the factors influencing turbidity development:

(1) pH - turbidity development is maximum at pH 3.8

(2) ionic strength - increasing salt concentration causes a marked drop in turbidity.

The details of the Schering method, with minor modifications, follow. This procedure was used in all quantitative determinations made in the present investigation. All pH determinations were made by the glass electrode.

1. Preparation of Solutions

(a) Buffer Solutions

0.1 acetate - pH 6.0 - in 0.15 M Nacl

0.5 acetate - pH 4.2

(b) Potassium Hyaluronate

Preparation is dissolved in 0.1 M acetate-sodium chloride buffer, pH 6.0 so that concentration in assay will give a transmission of 50 ± 5 per cent at a wave length of 600 mμ.

A standard curve to show turbidity development between hyaluronate and acidified protein showed that a concentration of 0.2 mg/ml buffer gives transmission of 47 per cent. Dilutions of substrate ranging from .06 mg/ml to 0.2 mg/ml showed absorbance proportional to concentration of substrate. Therefore the concentration of 0.2 mg hyaluronate per ml of buffer was used. As the solution ages, the turbidity obtained appears to decrease slightly. The solution is kept in the cold and discarded after 3 weeks. Potassium hyaluronates supplied by the Schering Corporation and by the Wyeth Institute were used.

(c) Hyaluronidase

Specimen to be assayed is dissolved at room temperature in pH 6.0 acetate-sodium chloride buffer. Concentration is chosen so that 1 ml contains approximately 3 units. Enzyme solution is used immediately after being made.

(d) Acidified Protein Solution

Horse serum is diluted 1 to 10 with 0.5 M acetate buffer, pH 4.2. pH is adjusted to 3.10 with 4 N hydrochloric acid. This solution is placed in tubes which are immersed in a boiling-water bath for 30 minutes. The solution is cooled and filtered. It can be kept in cold for at least two weeks.

2. Incubation of Enzyme and Substrate

Coleman tubes are set up for each assay as shown:

Tube No.	ml Substrate Solution	ml pH 6.0 Ac-Cl Buffer	ml Enzyme Solution
1	0.50	0.50	---
(lower blank) 2	0.25	0.75	---
3	0.50	---	0.50
4	0.50	1.10	0.40
5	0.50	0.20	0.30
6	0.50	0.30	0.20
7	0.50	0.40	0.10
8	---	1.00	---

Solutions are mixed and tubes are immersed in a constant temperature water bath at 37.5C for 30 minutes. To inactivate the enzyme the tubes are immersed in 60C water bath for 10 minutes. They are cooled to room temperature. No more that 3 enzyme fractions and the blanks are assayed simultaneously.

3. Development of Turbidities

To each tube are added 3 ml of 0.5 M acetate buffer, pH 4.2, followed by 1 ml of acidified serum. The contents are mixed thoroughly. After 5 minutes the turbidities are read in the Coleman Junior Spectrophotometer at wave length of 600 mμ. The instrument is set to give 100 per cent transmittance with tube #8.

4. Method of Calculation

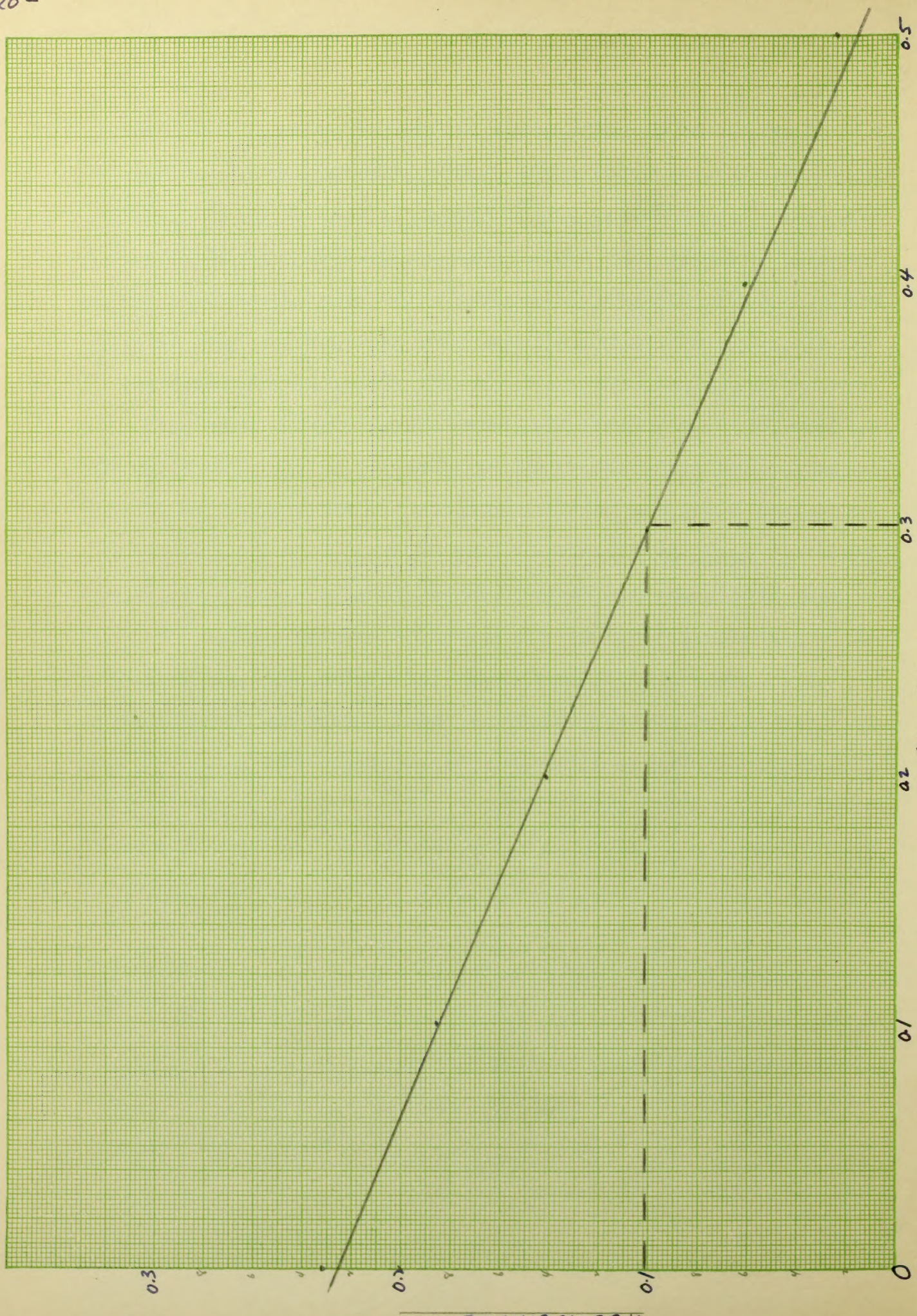
Turbidity readings (in terms of absorbance) are plotted against concentration of enzyme. This should give a straight line. Enzyme concentration corresponding to turbidity of lower blank is read from graph. This indicates the amount of enzyme containing one turbidity reducing unit.

Kass and Seastone (37) arbitrarily defined one turbidity reducing unit (TRU) as that amount of enzyme which in 30 minutes will reduce the turbidity produced by 0.2 mg of hyaluronic acid to the equivalent of the turbidity produced by 0.1 mg. It appears that the unit of the Schering Method is based on this definition.

A search of the literature for references concerning non-enzymatic factors which might depolymerize hyaluronate shows that the culture media contain nothing likely to cause false results. Favilli (19) found that an azoprotein prepared from diazobenzenesulphonic acid coupled with horse serum would reduce the viscosity of synovial fluid. The rate differs from that of hyaluronidase and pH has very little effect on the reaction. Madinaveitia and Quibell (45) found that ascorbic acid and certain diazo compounds could cause a fall in the viscosity of hyaluronic acid, but that the reaction is independent of pH. Robertson et al (67) found that ascorbic acid in the presence of H_2O_2 brings about a degradation of synovial mucin; but this is not accompanied by the liberation of reducing sugar.

As a precaution a few "blanks" of sterile media were run for possible turbidity reduction. None showed any reduction.

A representative assay is shown on pages 20-21.



REPRESENTATIVE ASSAY

Filtrate Dilution Factor - 15
(Filtrate 1 ml
pH 6.0-Ac.-cl buffer 14 ml)

Tube No.	ml Substrate	Conc. Substrate mg/ml	ml pH-60 Ac-cl Buffer	ml Diluted Filtrate	ml pH 40 Ac Buffer	ml Acidified Serum	Per Cent Transmission	Absorbance (2-log Transmission)
1	0.50	0.2	0.50	---	3	1	58^{3}	.2310
2	0.25	0.1	0.75	---	3	1	78^{0}	.1079
3	0.50	0.2	---	0.50	3	1	94^{0}	.0269
4	0.50	0.2	0.10	0.40	3	1	87^{0}	.0605
5	0.50	0.2	0.20	0.30	3	1	79^{1}	.1010
6	0.50	0.2	0.30	0.20	3	1	72^{1}	.1412
7	0.50	0.2	0.40	0.10	3	1	65^{1}	.1855
8	---	---	1.00	---	3	1	---	---

Calculation:

From graph: 0.302 ml diluted filtrate contains 1 TRU

∴ 1 ml diluted filtrate contains 3.3 TRU
Dilution factor = 15

∴ 1 ml filtrate contains 50 TRU

3. Production of Hyaluronidase by the Staphylococcus

A modified tryptic digest pH 7.6 was selected as the culture medium for the following reasons:

(1) It has been established by Rogers (68) that optimal formation of hyaluronidase occurs only in well buffered media.

(2) Previous studies of staphyloccic coagulase production, made at Boston University School of Medicine have shown that this medium is highly satisfactory for the growth of the staphylococcus.

Four strains of S. aureus were assayed quantitatively for hyaluronidase production:

L-isolated 4-46 from a mastoid infection, known to be hemolytic and to produce coagulase in large amounts.

Lewis-isolated 4-48 from a furuncle; known to be hemolytic and to produce coagulase in moderate amounts.

209-Department of Agriculture stock culture used for testing disinfectants; known to be non-hemolytic and a non-producer of coagulase.

78-Massachusetts Department of Public Health strain, isolated as a cause of food poisoning; known to be hemolytic and to produce coagulase in small amounts.

0.1 ml portions of 18 hour broth subcultures from stock slants, were inoculated into 100 ml portions of tryptic digest broth and the latter incubated for one week at 37C. The cultures were centrifuged at high speed at 8C for 45 minutes and the supernatant passed through a Mandler #6 filter. The filtrate wasassayed by a turbidimetric method, the details of which appear under Quantitative Assay of Hyaluronidase. Results were as follows:

L-Hyaluronidase present, the best preparation containing 137 TRU per ml.

Lewis-No hyaluronidase present.

209-No hyaluronidase present.

78-Hyaluronidase present, the only 100 ml portion of filtrate assayed showing 40 TRU per ml.

Eleven other strains of S. aureus recently isolated from hospital cases, but whose histories were unavailable, were assayed qualitatively by the following procedure:

5 ml portions of broth were inoculated from blood agar plate cultures of each strain,and incubated at 37C. The broth supernatant was tested after 24 hours and again after 48 hours if the 24 hour culture contained no enzyme. 0.5 ml portions

of the supernatant undiluted, diluted 1-5, and diluted 1-10 with pH 6.0 acetate-choride buffer were added to 0.5 ml of potassium hyaluronate solution. Subsequent incubation and turbidity development followed the usual quantitative method. The degree of turbidity reduction of each dilution was recorded as:

3 plus - No turbidity present

2 plus - Very slight turbidity present

1 plus - Moderate turbidity present

0 - Turbidity present equal to that of the usual tube #1. (See under Quantitative Assay of Hyaluronidase.)

Of the 11 strains tested:

8 produced no hyaluronidase in 48 hours.

3 produced hyaluronidase in 24 hours.

The results are tabulated below:

Strain	Supernatant Undiluted	1-5 Dilution of Supernatant	1-10 Dilution of Supernatant
E. and E. 4	3 plus	3 plus	1 plus
C. H. 2	3 plus	2 plus	0
E. and E. 11	2 plus	1 plus	0

Strains 4 and 2 were inoculated into 100 ml portions of the medium and incubated at 37C for one week. Their growth was

like that of the L strain, to be described subsequently.

Cunliffe et al (72) studied over 800 strains of staphylococci and micrococci. They found that almost 90 per cent of the coagulase positive group were also positive for hyaluronidase, as demonstrated by the mucin clot prevention test. Most of the deficient organisms were isolated from normal carrier sites or apparently healthy wounds. Of 160 coagulase negative strains none produced hyaluronidase.

In order to obtain some idea of when the enzyme was produced, and of its stability in the medium at 37C, the following was done:

One 100 ml portion of medium was inoculated with the L strain, as previously described. At 48, 96 and 144 hours, after inoculation 5 ml samples were withdrawn, centrifuged and the supernatant assayed for hyaluronidase. Results were as follows:

48 hour sample-39 TRU/ml

96 hour sample - 45 TRU/ml

144 hour sample - 75 TRU/ml

These values are only approximations due to the fact that complete removal of the bacteria from the supernatant was not possible, causing a slight cloudiness not desirable when a spectrophotometric method is used.

It is of interest to note that at the end of one week 6 separate portions of medium inoculated with equal amounts of an apparently homogenous suspension of the seed cultures, and

grown under the same conditions sometimes show wide variations in their hyaluronidase content, as determined qualitatively. The reason is difficult to explain. Possibly it could be due to mutant forms that outgrow the smooth forms.

At one point in the investigation the stock L strain roughened. Coagulase production was negligible and no hyaluronidase was demonstrated. Conversion to the smooth form with simultaneous satisfactory production of coagulase and hyaluronidase was accomplished by growing the organism in blood broth for a week, transfers being made every 24 hours. It is of interest that Duran-Reynals (17) reported that extracts of S. aureus typical R variants with rough colonies showed no spreading factor.

Assays of the L supernatant made immediately before and after filtration show that passage through a #6 Mandler filter produces no change in enzyme activity.

Duran-Reynals (18) recommended extraction with 10 ml of water of a 24 hour agar slant of a staphylococcus, subsequent removal of the bacteria present and the determination of spreading factor present in this extract. Haas (26) mentioned that hyaluronidase appears in the culture medium during growth of the staphylococcus and that it remains in the solution when the organisms are removed by centrifugation. These references raised the question of whether there is any intracellular hyaluronidase in the staphylococcus and the following procedure was used with the hope that it would provide an answer.

The sediment obtained from the centrifugate of an L culture whose supernatant showed the presence of hyaluronidase was drained of the supernatant as completely as possible, washed with saline solution at 5C and centrifuged at 5C for 30 minutes. The supernatant was discarded and the process repeated. The bacterial sediment was resuspended in approximately 15 times its volume of water, covered with an excess of toluene and incubated at 37C for 48 hours. The preparation was then centrifuged at 5C for 45 minutes, the supernatant autolysate pipetted from beneath the toluene and passed through a Mandler #6 filter. 1 ml of the filtrate was diluted with 4 ml of 0.1 M acetate, pH 6.0 containing 0.15 M NaCl, and the mixture assayed. No hyaluronidase was present.

The above results appear to indicate that, within the limitations of the assay used, there is no intracellular hyaluronidase in the staphylococcus.

4. Attempted Partial Purification of Hyaluronidase

The literature contains few references to purification methods for bacterial hyaluronidase. Meyer (60) reported on the precipitation of pneumococcic hyaluronidase by sodium flavianate. Rogers (69) obtained highly active and purified preparations of streptococcal and staphylococcal hyaluronidase. The bacterial culture media was mixed with kieselguhr and filtered through paper. It was then dialysed against tap water for twenty four hours in the presence of toluene. After adjustment of the dialysate to pH 5.6 $Fe(OH)_3$ precipitation

was employed. After centrifugation in a Sharples supercentrifuge the precipitate was eluted with 0.2 M Na_2Co_3, as many as five elutions sometimes being necessary. This procedure gives a 20-50 per cent yield.

Because of its relative convenience Meyer's method was attempted in the present study. The bacterial filtrate was adjusted to pH 3.7 with 1N H_2SO_4 and centrifuged in the cold after one hour at 8C. For each 20 ml of supernatant 1 ml of 4 per cent sodium flavianate (Naphthol Yellow S) was added. A yellow precepitate immediately resulted. After centrifugation this precipitate was suspended in water and .01 N Na OH added drop by drop until solution was just complete. Reprecipitation and resolution were twice repeated. Subsequent assay showed very slight activity:

Crude Filtrate	Flavianate Preparation
16.2 TRU/mg Nitrogen	5.0 TRU/mg Nitrogen

Possibly this low value can be attributed to denaturation of the enzyme by .01 N NaOH and to inadequate control of ionic concentration. However, assay of the flavianate supernatant showed hyaluronidase present and assay of the pH 3.7 precipitate showed considerable activity. In view of the latter finding isoelectric precipitation was attempted, using the following procedure.

Each of six 20 ml portions of a filtrate obtained from a six day culture of the L strain was adjusted to a desired pH by the addition of 1N HSO_4. The pH values were determined by

the glass electrode. The pH of the filtrate before any addition of acid was 7.8. The first 20 ml portion was taken to pH 5.5, the second to pH 5.0, the third to pH 4.5, the fourth to pH 4.0, the fifth to pH 3.5 and the sixth to pH 3.0. All portions were set aside at 8C for one hour and centrifuged in the cold for fifteen minutes. The supernatant obtained was clear. Each sediment was drained of its supernatant and dissolved in 20 ml of Na_2Co_3 solution, pH 7.8. This was made by adjusting the pH of 0.1 M Na_2Co_3 with 0.5 M acetic acid until the pH meter registered 7.8. The pH of each sediment was checked by the glass electrode and adjusted to 7.8 with a drop of 0.5 M acetic acid when necessary. The dissolved sediments were assayed quantitatively, the supernatant qualitatively. To rule out the possibility that apparent turbidity reduction of the supernatant might be in reality a failure to produce turbidity, caused by increased ionic strength and an unfavorable pH, and equal amount of unincubated supernatant was tested. This method showed turbidity development comparable to that of the upper blank, described under Assay. Thus it is assumed that turbidity reduction, if present, is due to enzyme action. Nitrogen present in the dissolved sediments was determined by the micro-Kjeldahl digestion method of Wong, followed by Koch-McMeekin Nesslerization. The results obtained follow:

pH	Enzyme Units/ml	Nitrogen mg/ml	Ratio units/mg. nitrogen	Qualitative test of Supernatant 1-10 Dilution
7.8-original filtrate	106	2.6	41	3 plus
5.5-sediment	N.S.Q.	(0.002)	--	3 plus
5.0-sediment	2.4	.004	600	3 plus
4.5-sediment	3.1	.069	45	3 plus
4.0-sediment	10	.067	149	3 plus
3.5-sediment	11	.078	141	3 plus
3.0-sediment	35	.132	265	3 plus

These data indicate the necessity of a quantitative study of the supernatant portions, using adequate pH and ionic control. The fact that an enzyme which is active at pH 7.8 does not show more complete precipitation at pH 3.0 seems somewhat unusual and suggests the possibility of the existence of more than one enzyme. This postulation has been made by Rogers. (69).

Isoelectric precipitation appears to be one means of concentrating the enzyme. It is likely that better preparations could be obtained by:

(1) Repeated thorough washing with water before the sediment is dissolved in the Na_2Co_3 solution.

(2) Dialysis of the dissolved sediment against tap

pH	Enzyme Units/ml	Nitrogen mg/ml	Ratio units/mg nitrogen	Qualitative test of supernatant 1-10 Dilution
7.0-original filtrate	106	2.5	41	3 plus
5.5-sediment	[illegible]	(0.002)	--	3 plus
6.0-sediment	5.4	.009	600	3 plus
4.5-sediment	5.1	.039	13	3 plus
4.0-sediment	10	.067	149	3 plus
3.5-sediment	11	.078	141	3 plus
3.0-sediment	36	.132	272	3 plus

These data indicate the necessity of a quantitative study of the supernatant portions, using adequate pH and ionic control. The fact that an enzyme which is active at pH 7.0 does not show more complete precipitation at pH 3.0 seems somewhat unusual and suggests the possibility or the existence of more than one enzyme. This postulation has been made by Rogers. (83).

Isoelectric precipitation appears to be one means of concentrating the enzyme. It is likely that better preparations could be obtained by:

(1) Repeated thorough washing with water before the sediment is dissolved in the Na2CO3 solution.

(2) Dialysis of the dissolved sediment against tap

water.

(3) Use of alcohol or dioxane to reduce solubility.

Subsequent lyophilization and preservation in a deep freeze should reduce deterioration. The dried material thus prepared should readily dissolve in pH 6.0 acetate-chloride buffer when it is required for assay.

III FINDINGS AND CONCLUSIONS

Using a turbidimetric method for assay it has been demonstrated that --

(1) Some strains of S. Aureus produce hyaluronidase when they are grown in a modified tryptic digest medium, under the usual conditions necessary for bacterial growth.

(2) The hyaluronidase - positive L strain shows repeatedly that no intracellular enzyme is present.

(3) The R mutant form of the hyaluronidase - positive L strain fails to produce hyaluronidase. Duran-Reynals (17) reported a similar observation in staphylococcal strains that had roughened.

(4) The hyaluronidase present in a bacterial filtrate of the L strain, at pH 7.8, can be concentrated and purified to some extent by means of partial precipitation at pH 3.0.

These findings appear to justify the following conclusions:

(1) The turbidimetric method of assay is satisfactory for the demonstration of the presence or absence of staphylococcal hyaluronidase in modified tryptic digest medium.

(2) There is a possibility of the existence of more than one staphylococcal hyaluronidase. This is in accord with the view of Rogers (69).

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V ABSTRACT OF THE THESIS

A review of the literature pertaining to hyaluronidase in general is presented, the references to staphylococcal hyaluronidase in particular being too few to provide an adequate background for this study. The method of culture of the staphylococcus and the preparation of a sterile filtrate satisfactory for hyaluronidase assay are described. The details of the measurement of hyaluronidase, based on the principle of turbidimetry, are given. The quantitative method used is that recommended by the Schering Corporation. The qualitative method was devised during the present investigation as a screening process, when a limited supply of potassium hyaluronate had to be considered. The results of the quantitative assay of four strains of S. aureus, grown under specified conditions, and the results of the qualitative assay of eleven strains of S. aureus, grown under specified conditions, are reported. An experiment designed to prove or disprove the existence of intracellular staphylococcal hyaluronidase is described and the result reported. The attempted partial purification of the enzyme by two precipitation methods is reported, the ratio $\frac{\text{turbidity reducing units}}{\text{mg nitrogen}}$ being used as an index of the efficacy of each precipitation. The results obtained are reported and their implications noted.